THE

FERMENTATION TUBE

WITH SPECIAL REFERENCE TO ANAEROBIOSIS AND GAS PRODUCTION AMONG BACTERIA

BY

THEOBALD SMITH, PH.B., M.D.

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In the study of the microscopic forms known as bacteria we have what might be fitly called the focal point of the various branches of biological science. Though their investigation may require careful morphological researches yet the unmistakable monotony of form, combined with a considerable variation of physiological activity, has compelled the bacteriologist to pay much attention to means by which such physiological variations may be more or less accurately registered in order that they may serve as a supplementary basis for classification. Again, with unicellular organisms the manifestations of cell activity become the most important phenomena for study. These manifestations bring together the fields of physiology and chemistry and make bacteriology in one sense a branch of physiological chemistry.

In dealing with bacteria and the results of their activity, one fact strongly impresses us and that is the necessity of knowing precisely and unmistakably the organism before us. No matter how profound the physiological and chemical studies of bacterial life, unless they are linked to an organism readily identifiable they have failed to assert their full value. In all the investigations of bacteria in their relation to the fermentation industries, to the dairy, to the soil, and to human and animal diseases now going on, the element of fundamental importance is the organism itself. About this all functions are grouped, to this every question finally reverts. The necessity for more accurate means of recognizing species and varieties has, however, not generally been felt and the methods of diagnosis have not kept pace with progress in the more practical

fields of microbiology. The species studied some years ago are assuming a more and more hazy outline and questions are constantly arising concerning the possible identity of old with new forms. This condition is largely unavoidable in a young and rapidly growing department of science and is in part due to the fact that investigators are too prone to attack new problems before the more orderly work concerning the old ones has been completed. For this state of affairs they are hardly to be blamed, for the profound relations of bacteria to other life on our globe has given the study of them a practical bias without which the resources now employed in investigations could never have been wrested from the utilitarianism of our present social organization.

It is due to considerations such as these that this article is presented as a contribution to the methods by which bacteria may be more definitely recognized. A complete differentiation is possible only through a complete knowledge of the biology of any given organism. This knowledge is only gradually acquired and more or less temporary expedients must be resorted to to fix the hosts of microörganisms shading into one another by almost intangible gradations of form and function. Among these expedients the fermentation tube occupies an important place in the differentiation of the more saprophytic forms and in giving us a fairly good conception of their powers of fermentation. I can do no better therefore in commemoration of the present occasion than to offer the observations which I have made with it during the past four years, as a connected whole to the biologist.

The fermentation tube appears to be an apparatus of considerable antiquity. The bent tube closed at one end has been used by chemists in storing small quantities of gas for qualitative analysis. I have been unable to determine who was the first to apply it to fermentation processes. In Detmer's *pflanzenphysiologisches Practicum* I find it figured as *Kühne'sches Gährungsgefäss.* More recently it has been adapted by Einhorn[1] for the quantitative determination of sugar in urine and by Doremus for the quantitative determination of urea in the same fluid. In 1889 I conceived the idea of using this tube as an ordinary culture tube in order to determine something more definite concerning the production of gases by bacteria without resorting to the complex manipulations of the chemist[2]. The form of the tube used in the following study

is given half size in the plate (fig. 1). It is essentially a tube bent at an acute angle, closed at one end and enlarged at the other into a bulb. At the angle the tube is more or less constricted. To it a glass foot is attached so that the tube may stand upright. For the sake of uniformity, the closed portion of the tube will be denominated "closed branch," the open portion, "the bulb," the intermediate narrow, bent portion the "connecting tube."

In the construction of this simple bit of apparatus several points must be borne in mind. The bulb should be large enough to receive all the fluid contained in the closed branch, for in some kinds of fermentation, the gas production drives out all the fluid from the closed branch. The cotton-wool plug must not be moistened under such circumstances otherwise the purity of the culture is imperilled. If the bulb is sufficiently large this difficulty will not arise. The connecting tube should not be too small, for then the filling and emptying of the closed branch becomes very tedious. Nor should it be too large, otherwise the anaërobic properties of the fluid in the closed branch, to be discussed farther on, may be less effective. Lastly the angle formed by the two branches of the tube must not be too acute otherwise the tube must be tilted so much during the transferrence of the fluid from the bulb to the closed branch that there is danger of its moistening the plug or even running out of the bulb. Since the closed branch is not accessible to cleansing with a brush it is advisable to fill the tube after use with the ordinary cleaning mixture (bichromate of potash and sulphuric acid) and allow it to stand undisturbed for some days.

The filling of the tube with culture fluid does not give rise to any difficulty. The fluid is poured into the bulb until this is about half full. The tube is then tilted until the closed branch is nearly horizontal so that the air may bubble up through the connecting tube and permit the fluid to enter the closed branch. When this has been completely filled, enough fluid should be added to cover the lowest expanding portion of the bulb. If the tubes are likely to remain unused for a month or longer it is best to add fluid until the bulb is half full to allow for evaporation.

The sterilization requires a few suggestions. This is best done in a steamer like the 'Arnold' for the tubes can be placed directly on the perforated plate in the bottom of the steam chamber. If a steamer is not at hand, an ordinary tin or granite-ware pail having a tight cover may be used. Enough water is poured in to form a shallow layer. To prevent the upsetting of the tubes by the ebullition I have been in the habit of placing them, three or four together, into perforated cups which are placed directly on the bottom of the pail. Steaming or boiling on three consecutive days is sufficient for complete sterilization. During the boiling the tension of the aqueous vapor in the closed branch

forces much of the fiuid into the bulb. As soon as the lid is removed the fluid returns to its former place in the closed branch with the exception of a small space at the top which is occupied by air originally dissolved in the liquid and driven out by the boiling. This air bubble should be tilted ont. After the second boiling some air may still be present. If this be tilted out the fluid will be found entirely free from air after the third or last boiling.

PHENOMENA OF ANAEROBIOSIS AND REDUCTION.

For the cultivation of bacteria the fermentation tube consists of two quite distinct portions sharply demarcated at the place indicated by the line *xy* in fig. 1. The bulb contains fluid in direct communication with the air while the fluid in the closed branch is almost entirely shut off from any such communication. Moreover, during the process of sterilization, the fluid in the latter has been entirely freed of air, as described above. This oxygen-free condition of the fluid is very clearly demonstrated by the following simple experiment :

If to peptone bouillon be added a few drops of a concentrated aqueous solution of litmus, methylene blue or indigocarmine, and fermentation tubes be filled with this colored fluid and sterilized, the fluid will be decolorized during the boiling by reducing processes due to the organic substances in the peptone bouillon*. In the open bulb the presence of air very speedily causes a return of the color. In fact it may not completely disappear at any time. If the tubes containing the colorless, reduced litmus or methylene blue be allowed to stand in a place sheltered from sudden changes of temperature, the fluid in the closed branch remains free from color (with perhaps a faint indication of color near the connecting tube) until the time arrives when the fluid in the bulb has evaporated and a bubble of air escapes into the closed branch.†

*I at first conceived the reducing action due to the glucose only, but the same process went on in peptone bouillon free from glucose. It is not due to simple boiling, however, for litmus or other coloring matter contained in simple bouillon or in water with or without a little Na_2CO_3 remains unchanged in the sterilization. It is thus dependent on the presence of glucose or peptone.

† This occurrence is like the escape of air into the reservoir of a student lamp which brings about the continuous feeding of the wick with oil.

Then the color begins to return and shows itself first at the very top of the closed branch beneath the air bubble. Thence it spreads slowly through the liquid as the evaporation continues to bring more air into the closed branch. These facts make it clear why the connecting tube should be as narrow as is compatible with the ready filling and emptying of the closed branch, for the smaller the calibre of this tube the less the interchange of fluid between open and closed portion.

Let us now consider the effect which this oxygen-free state of the culture fluid has upon the multiplication of bacteria. There is first of all a class of bacteria which multiply remarkably well in the bulb and the connecting tube but the fluid of the closed branch is shunned by them so thoroughly that it remains perfectly clear and limpid. The line of demarcation between the turbid, teeming liquid of the bulb and connecting tube, and that of the closed branch is sharply drawn. Evidently this class of bacteria are not only unable to multiply in fluids deprived of oxygen but they seem to avoid them as if influenced by a negative chemotaxis in spite of the power of motion which many of these forms possess. This limitation of growth has been observed in case of the same species from widely different sources as to time and place and hence stands for a constant character of the species. To this class belong many spore-bearing bacilli found in nature (*Bacillus subtilis*) and other forms (*Bacillus fluorescens liquefaciens*), and it corresponds to the class long known as the obligatory aërobic bacteria. The old test for this class, introduced by R. Koch, was an incapacity to multiply under a mica plate laid upon the gelatine layer in which the bacteria were supposed to be multiplying.

A second group of bacteria multiply not only in the open bulb but also in the closed branch. The fluid becomes uniformly clouded but the growth soon subsides for there is in most cases a decided preference for the open bulb, varying slightly with different species. In this the density of the growth always corresponds to that of cultures in ordinary cotton-plugged test tubes containing the same fluid. To this

class belong the greater number of the gas-producing bacteria to be considered farther on. It corresponds to the facultative anaërobic group, that is, those forms which are capable of multiplying to a certain extent in media free from oxygen although they flourish best in the presence of this gas.

There is lastly a third group of bacteria, of which I have examined only a small number in the course of the past four or five years, which do not multiply in the open bulb but seek the closed branch. These are the strictly anaërobic forms which require a medium devoid of oxygen. Many of them are gas-producing.

The fermentation tube thus informs us at once to which of these three groups of bacteria any given species belongs. This determination is especially valuable with the facultative anaërobic and the aërobic species. The anaërobic nature of any given form is usually manifested beforehand by its refusal to multiply in the ordinary culture tubes. It is needless for me to go over the various methods and devices which have been and are still employed in defining the aërobic or anaërobic character of bacteria. They are given in part in current text books. The simplicity of the test in the fermentation tube will at once appeal to all who have striven to produce a vacuum or substitute for the air an atmosphere of hydrogen.

The possibility of cultivating aërobic and anaërobic bacteria in the same kind of tube makes more simple certain bacteriological work carried on hitherto under considerable difficulty and with but partial success. In the determination, both quantitative and qualitative, of bacteria in the soil or the intestinal tract for instance, the aërobes and anaërobes had to be dealt with separately. In the solution of such problems the fermentation tube may do good service if the method of dilution be employed. Since this tube shows no discrimination between these two physiological groups of bacteria all would have an opportunity to develop. I am well aware of the difficulties inherent in the method of dilution,—the difficulty of gauging the dilution beforehand, the large number of tubes required, the care to be taken in the manipulation of the fermentation tubes, their size and cost—but these diffi-

culties are not those which threaten the success of the work and they count for little in important special investigations.

The Reducing Action of Bacteria.—It has been known for some years that certain bacteria have the power of abstracting oxygen from compounds which hold it very loosely. It has been customary among bacteriologists to demonstrate this deoxidizing or reducing activity by adding certain substances to the culture fluid which are colored in the oxidized state but which lose their color in the reduced state. Among the substances used are those mentioned above in the discussion of the anaërobic properties of the tube, and the action of bacteria correspond precisely to the action of heat in the presence of glucose or peptone as has been already described. It is not my intention to discuss this interesting phenomenon of reduction among bacteria excepting to call attention to the fermentation tube in bringing it out.

It will be remembered that when methylene blue, or indigo carmine or litmus be added to peptone bouillon with or without glucose so that the fluid becomes distinctly colored, and the tubes steamed, the fluid in the closed branch becomes decolorized. If bubbles of air be tilted into the closed branch and out again repeatedly, the color returns. Such tubes inoculated with any bacteria which are capable of growing in the closed branch, if only to a slight degree, become within 24 hours completely decolorized, with the exception of a shallow layer of fluid in the bulb. In the closed branch, for reasons already given, the fluid remains indefinitely decolorized. In the bulb the color returns when for any cause the growth ceases and subsides. It is interestiug to note that in an ordinary bouillon culture of *B. coli*, the phenomena of reduction and oxidation could be witnessed for 15 days at the end of which period the culture was rejected. The methylene blue would lose its color within half an hour after it had been re-oxidized by allowing air to bubble up into the closed branch. If a small quantity of air was allowed to remain in the closed branch, a stratum of blue fluid would remain at the top of the fluid column near the air for some days, then disappear completely, thereby indicating the complete consump-

tion of the oxygen admitted to the confined space, by the vital activity of the bacteria. Again in glucose bouillon inoculated with hog cholera bacilli, the complete paralysis of the bacteria after a certain stage in the fermentation is very clearly demonstrated by a permanent return of the color of the fluid in the bulb. The contrast between the deep blue color in the latter and the yellowish hue of the decolorized fluid of the closed branch is very striking.

THE PRODUCTION OF GAS BY BACTERIA.

Attention has been called to the formation of gas by bacteria by a number of writers in the past. Thus Escherich[3] in 1885 demonstrated the fact that *B. coli* and *B. lactis aërogenes*, both bacteria of the intestinal tract described by him for the first time, produce gas in solutions of glucose and lactose. In 1886, Arloing[4] called attention to the same subject. The property of gas production had been long associated with the pathogenic bacillus of "black quarter" in cattle (*Rauschbrand, charbon symptomatique*) which produces gas in the tissues of the affected part. Similarly the anaërobic bacilli of tetanus and of malignant œdema are known as gas producers. Among these anaërobes the formation of gas was demonstrated by distributing the bacteria in deep layers of liquid agar containing glucose and congealing the agar at once. The formation of numerous gas bubbles throughout the agar and the breaking up of the jelly by large quantities of gas is described and pictured and is now a common sight in bacteriological laboratories. A large number of bacteria belonging mainly to the group of facultative anaërobes, are now known as gas producers. Nevertheless the production of gas by bacteria has not thus far been taken as a serious matter by bacteriologists in the differentiation and fixation of species and varieties. Many have of late years been in the habit of recording the presence or absence of gas in cultures, but by methods likely to mislead. Since the gas test has proved the only final means of differentiating two important species, *B. coli communis* and *B. typhosus*, much more attention has been paid to this function but the methods have not materially improved.

In spite of the fact that I called attention to this matter three years ago[2] by describing a procedure for determining the production of gas as simple as the ordinary cultivation of bacteria, this procedure has not been generally adopted largely because the fermentation tube itself seems to be looked upon as something beyond the range of the ordinary bacteriological outfit.

In referring to gas formation writers have been in the habit of calling attention to the gas bubbles which make their appearance under certain conditions in stick (*Stich-*) cultures in gelatin and agar as well as in inclined or "slant" cultures of agar if there is condensation water present. These bubbles appear in the depths of the gelatin, one or more days after inoculation, as flat, lenticular spaces cleaving the jelly in one or more directions. In agar stick cultures, kept at 37° C. they appear frequently within 24 hours after inoculation within the depths of the agar jelly. In slant cultures they are usually found between the agar and the sides of the tube, imprisoned there by the condensation water which fills the gap between the slightly retracted agar and the glass. These bubbles depend for their presence on two things: 1, The capacity of the particular species for fermenting glucose with the production of gas; and, 2, The presence of glucose in the meat used in the preparation of the nutrient gelatin or agar. As I shall point out farther on the meat infusion is in some cases entirely free from such fermenting substance and if accidently used the bubbles will not appear. This test is therefore unreliable. A much better method and one which should not be neglected if the fermentation tube is not at hand is to add a definite quantity of glucose (or some other carbohydrate) to the gelatin or agar. Gas bubbles will invariably appear if the species is capable of producing gas at all. So far as my observations have gone they show that all gas production is linked to the presence of glucose or some other carbohydrate in the culture medium. Before giving illustrations of this process among different bacteria a few remarks on the manipulation of the fermentation tube are in order.

The fluid used in all cases, with exceptions to be mentioned, was peptone bouillon containing either glucose, lactose or

saccharose. The bouillon was prepared by digesting fresh beef in water at 60° C. for several hours then filtering and adding ¼ per cent. peptone, ½ per cent. sodium chloride and about 3cc. of a normal solution of sodium carbonate for every hundred cc. of the fluid. This suffices to make it feebly alkaline. To this peptone bouillon 2 per cent. of one or the other of the three sugars mentioned was added and the resulting fluid sterilized in the fermentation tubes.

These are kept, after inoculation, in the thermostat at 37° C. A mark made on the sides of the closed branch at the end of every 24 hours with a glass pencil furnishes an approximate record of the rate of gas production. Unless this is done it is impossible to know precisely when the formation of gas is at an end and also whether or not the volume of gas has been diminished by absorption. It is best to wait 4 or 5 days after the production has ceased before making a final examination. This is done by noting the condition of the growth, the reaction of the fluid in the bulb* and the maximum quantity of gas produced. This is most easily done by laying directly on the tube a glass millimeter rule and noting the tube length occupied by gas. The entire length of the closed branch is also noted, making due allowance for the upper convex extremity and the lower constriction. This mode of measurement is sufficient since only comparative values are desired. For the same reason all barometic and thermometric corrections are omitted in these approximate estimations.

The examination of the gas produced was limited to the determination of the quantity of carbon dioxide and of the explosive character of the gas remaining after the absorption of CO_2 by sodium hydrate. These facts are determined by the following simple manipulations:

The bulb is completely filled with a 2 per cent. solution of NaHO and closed tightly with the thumb. The fluid is shaken thoroughly with the gas and allowed to flow back and forth, from bulb to closed branch and the reverse several times

*The reaction was noted by placing a drop of the fluid on delicate litmus paper. The cultures were occasionally boiled to drive off any CO_2. In no case did the reaction with the litmus paper change.

to insure intimate contact of the CO_2 with the alkali. Lastly, *before removing the thumb, all the gas is allowed to collect in the closed branch* so that none may escape when the thumb is removed. If CO_2 was present, a partial vacuum in the closed branch causes the fluid to rise suddenly when the thumb is removed. After allowing the layer of foam to subside somewhat, the glass scale is again applied to the closed branch and the amount of CO_2 absorbed may thus be measured. In all cultures of this character thus far examined the gas remaining was explosive in character and probably hydrogen. At any rate wherever hydrogen is referred to hereafter, it simply signifies an explosive gas whose analysis must be left to the chemist. The explosive character of this residue is easily demonstrated as follows :—The cotton plug is replaced and the gas in the closed branch allowed to flow into the bulb and mix with the air there present. The plug is then removed and a lighted match inserted into the mouth of the bulb. The intensity of the explosion varies with the quantity of air present in the bulb.

One difficulty with the culture fluid employed needs to be mentioned at the outset. It is the presence of a small, but variable amount of glucose in the beef or other meat employed. When only glucose is used the difficulty disappears, but when other sugars are used we are at a loss to know how much of the gas to ascribe to the glucose originally present in the beef infusion or even to know whether the other sugars added are at all attacked by the bacteria. Recently I tested beef broth as it was prepared from time to time in the laboratory by inoculating fermentation tubes filled with it with a variety of gas-producing bacteria. In the following table the total amount of gas is calculated in percentages of the total volume of the closed branch which is about 20 ccm. The amount of CO_2 absorbed by potash is given in percentages of the total volume of gas. The gas remaining is explosive.

I.—Total Gas Set Free in Meat Broths, etc., by Various Gas-Producing Bacteria.

Culture Fluid.	*B. cloacæ.*	*B. lactis ærogenes.*	*B. coli.*	*B. choleræ suis.*	*Proteus vulgaris.*
Peptone bouillon (beef) A.	. . . . .	33 pr ct. (CO_2=30 pr ct.)	27 pr ct.* (CO_2=33 pr ct.)	. . . . .	. . . . .
" " " B.	14 pr ct. (CO_2=11 pr ct.)	. . . . .	. . . . .	10 pr ct.	1 pr ct.
" " " C.	22 (CO_2=15)	. . . . .	15 (CO_2=22)	. . . . .	2.5
" " " D.	0	0	. . . . .	0	. . . . .
" " " E.	27 (CO_2=16)	. . . . .	. . . . .	. . . . .	. . . . .
" " " F.	22, 23, 24 (CO_2=17)	. . . . .	. . . . .	. . . . .	. . . . .
" " " G.	19	10	. . . . .	. . . . .	. . . . .
" " " H.	12	. . . . .	. . . . .	4	. . . . .
" " " I.	0	0	. . . . .	. . . . .	. . . . .
" " " J.	. . . . .	. . . . .	22 (CO_2=37)	. . . . .	. . . . .
" " (veal) . . .	26	. . . . .	. . . . .	. . . . .	. . . . .
Bouillon (pork)	2	. . . . .	. . . . .	. . . . .	. . . . .
Dunham's solution	. . . . .	0	0	0	. . . . .

* See fig. 8 of Plate.

This table shows that of ten samples of beef broth two were manifestly free from glucose. Hence the advice of Dunbar[5] to use simply beef infusion (*Fleischwasser*) to test the gas producing power of bacteria would lead to conflicting results unless glucose were added. That the sugar contained in muscular tissue is glucose as affirmed by physiologists seems to be borne out by the fact that it is attacked by bacteria which do not ferment lactose or saccharose.

In order to eliminate the source of error introduced by the muscle sugar I tried a solution of salts reccommended by Fermi[6] and of the following composition:

$MgSO_4$	0.2	gram.
HK_2PO_4	1.	"
$(NH_4)_3PO_4$	10	"
Glycerin	45	"
Water	1000	cc.

In this solution the bacteria experimented with failed to multiply when peptone was added and the glycerin omitted. When both were present the fluid in the open bulb became fairly turbid but that in the closed branch remained practically free from any growth. Evidently the glycerin could serve as food only in presence of oxygen. When glucose was added gas appeared, but much more slowly and in much smaller quantity than in peptone bouillon with glucose. A comparison of results obtained with this artificial solution and peptone bouillon was not possible and further trials with it were abandoned.

It next occurred to me that the sugar in bouillon might be removed by allowing some gas-producing bacteria to multiply in the latter for a time. The bouillon might then be resterilized after a certain quantity of some sugar had been added and the fluid reinocculated with the species to be studied. This procedure was found successful so far as gas production is concerned, but it went on more slowly and apparently in a somewhat different way. Hence this method was given up.

Dunham's solution (1 per cent. peptone and ½ per cent.

sodium chloride in water) was also tried. Bacteria multiplied so feebly in it, however, that it also was abandoned.

The method finally settled on was to test each quantity of bouillon prepared in the laboratory. If any failed to give rise to gas in the fermentation tube it was set aside to be used exclusively with these tubes. Unfortunately most of the gas-production recorded in the tables following, took place in bouillon containing traces of glucose since the work could not be delayed. The difficulty has been partly overcome by keeping a record of the quantity of gas formed in the same bouillon to which no sugar was added.

In searching through the literature of this subject I find that the presence of glucose in bouillon has likewise been noted by Peré[7] and by Pane[8] in its bearing on the products of bacteria fermentation. The former considered it mainly in its relation to the initial acidity of cultures, a relation, to which I had already called attention in 1890[9]. Pane sought to determine the gas produced in peptone bouillon quantitatively by noting the number and the size of the gas bubbles in bouillon-agar when *B. coli communis* was mixed with fluid agar and this rapidly hardened by cooling. He likewise determined the amount of acid produced by the fermentation of this carbohydrate.

TYPES OF GAS PRODUCTION.

In my experience with the cultivation of bacteria in the fermentation tube a variety of hitherto unnoticed details have come to light. In arranging and classifying these I find more or less difficulty. It seemed perhaps the simplest plan to describe the gas production of a very common and much discussed species—*Bacillus coli communis*—and then to refer briefly to those species which belong to the same general group. The observations of others so far as they bear on the subject before us will be reviewed in a succeeding chapter.

B. coli communis.—It is not my intention to enter into detail concerning the characters of this somewhat notorious intestinal species. At present its main differential characters are accepted to be 1, motility ; 2, prompt coagulation of milk; and, 3, gas production in nutrient media containing lactose.

As regards motility it is interesting to note that it is more easily overlooked in bouillon cultures than when very recent colonies on gelatin or agar are examined in the hanging drop. There is moreover a considerable variation among cultures from different sources as to this property of motility. There are to be found all gradations from cultures in which a motile form may be seen only after prolonged searching, to those in which almost all individuals are in motion. As to the coagulation of milk there is likewise some variation in this function. Some years ago I isolated an unquestionable colon bacillus from the feces of an infant, which failed to produce coagulation of milk even after several weeks' sojourn in the thermostat. The same may be said of some cultures from animals. These facts show that the colon bacillus is by no means a well characterized species and the question arises how shall the various races be classified? The same thoughts have been expressed by other writers especially by Gilbert and Leon[10]. I believe that the properties of these races as manifested in the fermentation tube will serve as the best basis for a classification.

If we take for our culture a bacillus isolated from human feces and manifesting all the characters usually ascribed to *B. coli communis* we shall observe the following phenomena in the fermentation tube at 37° C.

In glucose bouillon within twenty-four hours the entire fluid has become clouded and a certain quantity of gas has accumulated in the closed branch. At the end of the second day more gas has formed. At the end of the third day a trifle more is present. After this very little if any is set free. The cloudiness promptly subsides and all growth is apparently at an end. The fluid in the bulb will be found markedly acid. This acidity is undoubtedly the cause of the sudden cessation of activity, for if it be promptly neutralized with a sterile solution of some alkali the fermentation starts again. It should be stated that in these observations no "acid-binding" substance, such as $CaCO_3$ has been added to the fermenting fluid. The following table gives in percentages of the tube length of the closed branch (*i. e.*, of the volume of the latter) the amount of gas formed by *B. coli* from various sources :

II.—*B. coli communis* in Glucose Bouillon. (Fig. 2 of Plate.)

B. coli communis.	Gas accumulated at the end of				CO_2.	H.
	1 day.	2 days.	3 days.	9 days at 20°–25° C.		
	pr. ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.
1. From human feces	46	58	62	57	37.2	62.8
2. " " "	30	45	54	45 (6th)†	37	63
3. " cattle.	44	52	62	58	37.2	62.8
4. " swine.	36	42	44	46	33	67
5. " water.	28	44	47	44	32	68
6. " "	34	47	54 (5th)†	45	31.5	68.5
7. B. of Grouse disease (Klein)*	43	51	53	51 (6th)†	34	66

*This culture came thus labeled from Kral's collection in Prague. It corresponded closely with Klein's description of the bacillus isolated by him from the organs of diseased grouse[11]. I include it in this table because it resembles *B. coli* in many respects and its action on the sugars is vigorous and equal to that of any variety of *B. coli* here represented. A comparison of the gas production in glucose, lactose and saccharose bouillon shows this function to be identical in character in all three.

† The figures refer to the number of days after inoculation of the culture fluid.

From the foregoing table it will be seen that the largest amount of gas is produced during the first twenty-four hours and that the gas itself is made up of CO_2, one volume, to an explosive gas, two volumes. During the past five years I have examined a large number of cultures of *B. coli* which I isolated from the intestinal contents of domesticated animals

and in every case this ratio of CO_2 to H was the same. The somewhat crude method of measuring the gas, the contraction of its volume when removed from the thermostat, the fluctuating temperature of the room, the presence of a layer

III.—*B. coli* IN LACTOSE BOUILLON. (Fig. 3 of Plate.)

B. Coli communis.	Gas accumulated at the end of							CO_2.	H.
	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.	Total at 20°–25° C.		
	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.
1. From human feces . . .	42	58	62	. . .	. . .	. . .	60 (11th)	33.8	66.2
2. " " " .	60	65	. . .	. . .	. . .	66	60 (7th)	. . .	. . .
3. " cattle	51	56	60	. . .	. . .	61	56 (7th)	36.7	62.3
4. " swine	39	44	46	. . .	51	. .	50 (8th)	40	60
5. " water	28	42	45	48	. . .	52	45 (8th)	37	63
6. " "	33	51	56	. . .	. . .	64	59 (11th)	40.5	59.5
6.* " "	24	34	44	48	50	. . .	48 (8th)	35	65
7. B. of Grouse disease	55	62	64	. . .	. . .	. . .	61 (6th)	37	63

* Bouillon entirely free from muscle glucose. Culture made six months after the first.

of foam on the surface of the liquid in the closed branch after the CO_2 has been absorbed, all these factors enter as slightly disturbing elements and make the values quoted as only approximately correct. As might be anticipated from the

prompt precipitation of the casein in milk inoculated with *B. coli*, this organism acts upon lactose in the same way as upon

IV.—*B. coli* IN SACCHAROSE BOUILLON. (Fig. 4 of Plate.)

B. coli communis.	Gas accumulated at the end of						Total gas at 20°-25° C.	Reaction of bulb.	CO_2	H
	2 days.	4 days.	6 days.	7 days.	9 days.	12 days.				
	pr ct	pr ct	pr ct	pr ct	pr ct	pr ct			pr ct	pr ct
1*	22.4	30	56	63	. . .	41 (11th)	60 (11th)	acid	38	62
1.	16	. . .	. . .	26	31	58 (13th)	65 (29th)	alkal.	43.5	57.5
2	21	38	46	. . .	53	. . .	63 (20th)	acid	37	63
3	. . .	. . .	. . .	. . .	bubble	. . .	. . . .	alkal.	. . .	. . .
4	18	21	26	27	32	36	46 (18th)	acid	38.2	61.8
5	31	47	50	. . .	. . .	. . .	43 (11th)	"	36.5	63.5
6†	7	13	15	16	20(8th)	. . .	19 (10th)	alkal.	23	77
6†	. . .	. . .	. . .	14	. . .	. . .	13 (10th)	"	20	80
7 (Grouse disease)	47	50	. . .	. . .	. . .	. . .	50 (6th)	acid	35.5	64.5

* These figures correspond to those of the cultures in tables II and III.
† Much of the gas set free by this bacillus must be ascribed to muscle glucose.

glucose and the phenomena in the fermentation tube containing lactose bouillon are precisely the same as those in glucose bouillon.

The action of *B. coli* on cane sugar in peptone bouillon dif-

fers with rare exceptions, quite markedly from that upon glucose or lactose. The examination of cultures from different sources has revealed two distinct varieties, one of which produces a considerable quantity of gas, the other little or none. With the former variety the type of gas production varies somewhat from culture to culture. In general the fluid is driven out very slowly and the gas production may last several weeks. These statements are well illustrated in table IV.

When the gas production goes on very slowly the growth in the open bulb becomes exceedingly abundant. This is most probably due to the slow neutralization of the bacterial alkali, formed in the open bulb, by the acid resulting from the slow fermentation in the closed branch. The gradual entrance of this acid fluid into the bulb acts as a continuous stimulant to the multiplication of bacteria there. When the gas production is rapid the fluid in the bulb remains acid and the growth speedily subsides.

What the true significance of the varying behavior of the *B. coli* group towards cane sugar is, can be determined only by more extended investigations. I venture to suggest however, that the saccharose fermentation may require in the slow fermentation the presence of an inverting ferment while that of lactose and glucose goes on without it. This ferment is apparently no longer formed by some bacteria otherwise not distinguishable from *B. coli*. The whole subject is very interesting and seems to indicate either that this species may readily lose the capacity to act on cane sugar or else that it is in a transition stage towards the more pathogenic species of this large group of bacteria. The peculiarity of the saccharose fermentation suggests the thought that the presumable ferment is formed only in the fluid in contact with oxygen and that it very slowly diffuses thence into the closed branch. A layer of sterile oil on the fluid of the bulb would perhaps answer this question. But I have had no opportunity to try this expedient. We may summarize the facts concerning the gas-producing power of *B. coli communis* briefly as follows :—

In feebly alkaline peptone bouillon containing 2 per cent. of glucose or lactose, about 50 to 60 per cent. of the closed branch of the fermentation tube will be occupied by gas in 3 or 4

days and the fluid will be strongly acid. The gas is composed of about 2 volumes of H and 1 volume of CO_2. In bouillon containing 2 per cent. cane sugar the gas production goes on in cultures of some varieties. It accumulates more or less slowly and the ratio of CO_2 to H varies.*

The Hog-cholera Group of Bacilli.—While forms differing more or less in physiological and cultural features are thrown together as *B. coli communis*, pathogenic forms having much closer affinities, in fact scarcely any points of difference, are carefully separated and named. This anomaly is due to the practical importance of pathogenic species. Of the hog cholera bacillus itself, an organism of considerable economic importance as well as of marked pathological interest, I have examined in the course of the past seven years a number of cultures from widely different regions of our country. Some of these possessed minor varietal characters, among which may be included a considerable variation of pathogenic power. With a few exceptions the gas producing phenomena are remarkably uniform. In case of these exceptions, one of them a culture now seven and a half years old, the gas production is somewhat reduced quantitatively. Whether this is an original peculiarity or a result of prolonged cultivation I am not prepared to state.

They all possess the power of fermenting glucose in precisely the same manner as *B. coli*, but they are incapable of producing gas in bouillon containing cane sugar and milk sugar. The absence of any action on milk sugar in this group is correlative with the absence of any power to coagulate milk. Even after weeks of sojourn in the thermostat and subsequent boiling, milk cultures remain fluid. In this group I also include a still unnamed bacillus from the genital passages of a mare, *B. enteriditis*, Gärtner[14], and *B. typhi murium*, Löffler[15]. These are the only ones which I have carefully examined. There are probably others, found in different countries, which belong to this group of pathogenic bacteria.

In the following table are included several distinct physiological varieties of the hog cholera bacillus :—

* The products of the fermentation induced by *B. coli* have been more or less exhaustively studied by Baginsky[12], Peré[7] and Scruel[13].

V.—Hog Cholera Group of Bacilli in Glucose Bouillon.

Species.	Gas present after				Total at 20°–25° C.	CO_2.	H.	Remarks.*
	1 day.	2 days.	3 days.	4 days.				
	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	
B. choleræ suis, I	22	35	37	40	37	34	66	Culture 7½ years old.
" " " II	35	51	56	58	54	37	63	Culture 3 years old.
" " " III	45	58	58	58	52	36	64	Feebly pathogenic variety ; 4 years old.
" " " IV	33	43	45	45	42	33	67	Culture probably 6 years old.
(Swine pest.) Bacillus from mare	12	50	58	61	55	36.5	63.5	Culture 2 years old.
B. typhi murium (Löffler,) . .	. . .	46	48	. . .	50 (8th)	35	65	Culture probably 2 years old.
B. enteriditis (Gärtner,) . . .	. . .	49	52	. . .	52 (8th)	30	70	Culture probably 5–6 years old.

* The age here indicated refers to the time which has elapsed since the species was obtained from a case of disease, or since it was discovered.

I have omitted from the above record four additional cultures of *B. choleræ suis*, three of which are identical with II and III so far as the quantity of gas produced is concerned; the fourth corresponds to IV in this respect. In all varieties of this sub-group the behavior in glucose bouillon is precisely the same. There is a rapid evolution of gas on the first and the second day ceasing promptly on the fourth or fifth. The growth subsides at the same time. The culture fluid becomes strongly acid.

The action of this entire group on saccharose and lactose in bouillon is negative and hence I omit any tabulation of the records. Unless the bouillon is free from muscle glucose there may have accumulated, after one or two weeks, a certain amount of gas corresponding to that developed in the same bouillon free from any additions. This may amount to 15 or 20 per cent. of the contents of the closed branch. A glucose-free bouillon recently tried remained free from any gas. That there is, in such tubes, no action on the sugar is proven by the feeble transitory acid reaction when gas is formed to a slight extent. This soon changes to an alkaline reaction in the bulb. When glucose is entirely absent the acid reaction fails to appear.

B. lactis aërogenes Escherich[3]. The cultures which I have tested differ from those of *B. coli* in certain minor but definite characters. They were non-motile and provided with more or less zooglœar or intercellular, but not viscid, substance often recognizable on the border of the hanging drop as a distinct capsule. When these bacilli are cultivated on solid media this capsular substance manifests itself by a regular spacing between the individual bacilli when these are massed together. The growth on potato is richer and of a paler yellow color than that of *B. coli*. The surface colonies on gelatin are usually fleshier than those of the latter and frequently resemble little pearly drops. In old bouillon cultures there is noticeable an even stronger fecal odor than that arising from similar cultures of *B. coli*. I give the above characterization mainly because the species does not seem to be any more stable in its minor characters than *B. coli*. The following table gives the gas production of the only culture thoroughly examined.

It will be noticed that saccharose is not affected. The gas formed was traced to muscle glucose in the bouillon.

VI.—*B. lactis aërogenes* IN SUGAR BOUILLON.

Kind of Sugar.	Gas present after					Total at 70° F.	CO_2	H	Remarks.
	2 days.	3 days.	4 days.	5 days.	6 days.				
Glucose . .	41	54	. . .	. . .	. . .	50 (11th)	35	65	Growth subsided; acid.
	60*	. . .	. . .	. . .	. . .	58 (7th)	. . .	. . .	" " "
Lactose . .	45	61	63	66	68	62 (11th)	38.5	61.5	" " "
	40*	47	50	. . .	52	53 (14th)	38.7	61.3	" " "
Saccharose	8	9	11	13	. . .	13 (11th)	6	94	Growth abundant; alkaline.
	8*	12	. . .	. . .	15	17 (13th)	20	80	" " "

* The second trials with the same kind of sugar were made fully half a year after the first.

The fermentation in so far as the gas production is concerned is precisely similar to that of the entire *B. coli* group.

Some years ago[16] I examined comparatively three slightly different bacteria obtained from the intestines of the pig. One of these corresponded very closely to the species above described but differed from it in producing an abundance of gas in saccharose bouillon. We probably have a number of

VII.—Bacillus of Friedländer.

Sugar.	Gas accumulated after						Total gas at 20°–25° C.	Reaction of bulb.	CO_2.	H.
	2 days.	3 days.	4 days.	9 days.	10 days.	11 days.				
	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.
Glucose . .	41	45	. . .	. . .	. . .	. . .	43 (7th)	acid.	32.7	67.3
Saccharose	35*	42	46	. . .	. . .	. . .	46 (9th)	acid.	32.8	67.4
	36†	40	43	46(6th)	. . .	. . .	45 (8th)	acid.	34.6	65.4
Lactose . .	6*	12	15	17	21	24	23 (17th)	alkal.	14.3	85.7
	trace.†	4	8	11	13	. . .	13 (10th)	feebly acid	trace.	nearly 100

* Bouillon containing a trace of muscle sugar, (H of table I.)
† Bouillon containing no muscle sugar, (I of table I.)

varieties which may be grouped under the specific name *B. lactis aërogenes* and which further fermentation studies may tend to define and separate. There is furthermore good reason for regarding this species very closely related to *B. coli.*

The bacillus of Friedländer*. This species has aroused considerable attention owing to the supposition prevailing at one time that it was the cause of pneumonia in man. With reference to both morphological and cultural characters it seems to bear the same relation to *B. lactis aërogenes* which the hog cholera bacillus bears to *B. coli.* From the above table it will be seen that this organism acts vigorously upon glucose and saccharose but only feebly on lactose.

The persistence of the gas-producing function of this species is well illustrated by the fact that three years ago the same culture gave the following result[16]:

Glucose, total gas 44 pr. ct.; CO_2 43.4 pr. ct., H=56.6 pr. ct.
Saccharose, " " 46 " " " 41 " " H=59 pr. ct.
Lactose, " " 19 " " " 21.8 " " H=78.2 pr. ct.

B. œdematis maligni. Of anaërobic species only a few have been cultivated in the fermentation tube. Some of these were derived from the bodies of animals and represented those "post mortem" bacilli quite invariably present some time after death especially in the carcasses of large animals. These were found gas-producing but no record was kept. In 1890[18] I isolated an anaërobe, probably identical with the bacillus of malignant œdema, from the organs of a pig. I append a somewhat incomplete record of gas production in the fermentation tube studied at that time which indicates a close relationship to the same process in the *B. coli* group:

* This bacillus has been studied from the chemical aspect by Frankland, Stanley and Frew[17].

VIII.—*B. œdematis maligni.*

Glucose.				Lactose.		Peptone bouillon without sugar.		Saccharose.	
Time required.*	Total gas	CO_2	H	Time required.	Total gas.	Time required.	Total gas.	Time required.	Total gas.
	pr ct	pr ct	pr ct		pr ct		pr ct		
12 days	80	31	69	3 days	80	4 days	4	2 days	0
13 "	60	33	67	. . . . .	. . .	. . . . .	. . .	. . . . .	. . .
12 "	63	34	66	. . . . .	. . .	. . . . .	. . .	. . . . .	. . .
8 "	86	. . .	. . .	. . . . .	. . .	. . . . .	. . .	. . . . .	. . .

* The notes do not state whether the total quantity of gas had not been formed before the time indicated.

Proteus vulgaris. This species has certain points of contact as regards morphology with the *B. coli* group. It differs in possessing very active peptonizing properties as manifested in gelatin. Its power of gas production is peculiar in that a smaller quantity of gas is formed than in cultures of *B. coli.* It likewise is peculiar in being unable to produce gas in lactose bouillon while its action in glucose and saccharose bouillon is the same. Repetition of the gas test at intervals and with

subcultures which had lost almost completely the peptonizing power gave the same result.

IX.—*Proteus vulgaris.*

Sugar used.	Gas present after						Reaction of bulb.	CO_2	H
	1 day.	2 days.	3 days.	5 days.	7 days.	Total at 70° F. in 9 days			
	pr. ct.	pr. ct.	pr. ct.	pr. ct.	pr. ct.	pr. ct.		pr. ct.	pr. ct.
Glucose . .	4	20	28	—	35	31	Acid	28	72
Lactose . .	2	5	—	8	10	10	Alkaline	Trace	Nearly 100
	0*	0	0	0	—	—	"	—	—
Saccharose.	6	20	30	34	36	33	Acid	39	61
	—*	24	36	32	33 (6th)	32 (6th)	"	33⅓	66⅔

* Bouillon I of Table I containing no muscle glucose. The rest is bouillon II, containing a trace.

The Bacillus-Cloacæ Type of Gas Production.—The types of gas production hitherto described present certain underlying characters which suggest a close relationship. These I group together as the *B. coli* type since it differs quite markedly from the type now to be described.

The species known as *B. cloacæ* was first described by E. O. Jordan[19] as coming from sewage. The cultures which I have ranged under this name have been obtained, with one exception, from water both polluted and unpolluted. The exception was reputed to have come from cornstalks. It is therefore a widely diffused organism whose true habitat I do not know, although I am strongly of the opinion that it is an organism living on decaying vegetable matter. If so, its name is unfortunate as it could not be regarded as a sewage bacterium strictly speaking. It is a small bacillus closely resembling *B. coli* in form and size and is actively motile. On gelatin the surface colonies appear at first as thin expansions with slightly irregular outline. Two or three days after the colonies have appeared, liquefaction sets in. This peculiar retardation of liquefaction is noteworthy and in general, it may be said, that the rapidity varies slightly from culture to culture and is gradually weakened during artificial cultivation. Milk I find coagulated only after seven or eight days. On potato a fleshy, pale yellowish, not characteristic growth appears after one or more days. I may state here that two varieties of this species have come under my observation which I designate provisionally α and β. For α, the bouillon becomes uniformly turbid, for β very feebly so with a tendency of the growth to form flakes somewhat like the flocculi of anthrax cultures. Evidently there is in β a greater tendency towards cohesion of the bacilli.

The gas production of *B. cloacæ* is very rapid in glucose and saccharose bouillon and slow in lactose bouillon.

X.—*B. cloacæ*, (Figs. 5 and 6 of Plate.)

Bacillus.	Amount of gas accumulated after					Reaction.	H.	CO_2.
	1 day.	2 days.	3 days.	4 days.	Total 20°–30° C.			
α.	pr ct.	pr ct.	pr ct.	pr ct.	pr ct.		pr ct.	pr ct.
Glucose . .	32	77	. . . .	96	96 (4th)	faintly acid	30	70
Saccharose	70	95	. . . .	. . . .	86 (4th)	faintly acid	42	58
Lactose . .	15	20	. . . .	25	60 (22d)	alkaline	63.4	36.6
β.								
Glucose . .	. . . .	. . . .	73	78	82 (6th)	. . . . .	31.6	68.4
Saccharose	2	33	49 (4th)	73 (5th)	95 (7th)	acid.	25	75
Lactose . .	. . . .	20	. . . .	37	80 (10th)	acid.	46.5	53.5

This type of gas production differs from the *B. coli* type : 1, in the much greater accumulation of gas which drives out all of the fluid from the closed branch in two or three days ; 2, in the much larger proportion of CO_2, the fraction $\frac{H}{CO_2}$ varying from ½ to ⅓ ; 3, the much feebler acid reaction of the fluid in the open bulb. The lactose fermentation goes on at a slow steady pace and after one or two weeks a considerable quantity of gas has accumulated in which the relative quantity of CO_2 and H varies considerably.

The behavior of *B. cloacæ* in the fermentation tube reminds us of the action of ordinary yeast under the same conditions. There is in both the same rapid evacuation of fluid from the closed branch. The fundamental difference between the two processes, however, is the invariable presence of H in cultures of the bacterium.

XI.—*Saccharomyces cerevisiæ* (Fig. 7 of Plate.)

Sugar.	Gas accumulated after							Reaction.	CO_2	H
	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.				
	pr ct	pr ct	pr ct	pr ct	pr ct	pr ct			pr ct	
Glucose	21	63	. . .	100	. . .	. . .		neutral	100	. . .
Saccharose	0	3	27	50	83	96		acid	100	. . .
Lactose	0	0	0	0	0	0		alkaline, growth very feeble	. . .	. . .

Among the more important bacteria which have been tested in the fermentation tube and which fail to set free any gas may be mentioned the following:

Staphylocci.

Streptococci.

Septicæmia hæmorrhagica (rabbit septicæmia, swine plague, fowl cholera, *Wildseuche*, etc.)

B. typhi abdominalis.

The various comma bacilli (*Spirillum chol. Asiat.; Sp.* Deneke, Finkler and Prior, Smith.)

B. anthracis.

Many aërobic spore-bearing bacilli.

B. mallei.

Concerning that strictly aërobic species, *B. subtilis*, Vandevelde[20] finds, contrary to earlier determinations of Prazmowski[21], CO_2 and H given off in varying quantities. Obviously the former worked with impure cultures. The absence of gas production in cultures of *B anthracis* was pointed out by Arloing[4] in 1886.

SOME GENERAL OBSERVATIONS ON THE PRODUCTION OF GAS BY BACTERIA AND ITS RELATION TO THE FORMATION OF ACIDS IN THE CULTURE FLUID.

A consideration of the results obtained with the fermentation tube develops a number of interesting phases of bacterial life. Perhaps the most important fact to be gathered is the fundamental character of gas production not only in distinguishing species but groups of species. The phenomenon of fermentation as expressed by gas production may in fact be called a group reaction. It is, for example a common character of a large group of motile bacteria which we may designate the *B. coli* group. While it is absent in other equally large and important groups such as *Septicæmia hæmorrhagica* and the comma bacilli. I regard, therefore, the production of gas not as one of the large number of minor differential characters by which we are in the habit of fixing a species but as one of fundamental importance, associated with groups of bacteria having perhaps a common phylogenetic origin.

In view of the presumable importance of gas production* the question may be asked as to the permanence of this function. The permanent or temporary character, under cultivation, must largely decide for or against the position taken above as to the fundamental importance of kinds of fermentation in the grouping of bacteria. The facts which I have collected are necessarily meager since I have employed the fermentation tube only for four years, and no other person has thus far paid any attention to this subject. A few facts, however, bear on this point. I have not yet encountered any bacteria which have either gained or lost the gas-forming function under cultivation. In the colon group it does not appear to vary at all from year to year. The same persistence was observed in *Proteus vulgaris*. Of two varieties originally descended from the same colony, one still actively liquefying gelatin, the other having lost this power almost absolutely, both produce the same amount of gas in glucose and saccharose bouillon. Recently I have noticed in one of the cultures of *B. cloacæ*, over a year old, a slight diminuition in the total quantity of gas set free in saccharose bouillon. In glucose bouillon the function seems to be intact. While, therefore, the power of gas production may be slightly reduced quantitatively it does not disappear. It likewise is, at least for *Proteus vulgaris*, a much more permanent function than that of secreting a liquefying ferment.†

More or less related to an enfeeblement of the fermenting power observed in the space of months and years in the same culture, is an incapacity probably the result of an adaptation to a parasitic existence. This is very well illustrated by the

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*I simply use this word as standing for types of fermentation which need more careful examination by chemists than they have hitherto received.

†In opposition to my observations is one recorded by Arloing[1]. A *micrococcus septicus puerperalis* (probably a streptococcus or a staphylococcus) produces no gas when fluids containing sugar are inoculated from old cultures. When, however, young cultures twenty-four to thirty-six hours old are used for inocculation, CO_2 and H are given off abundantly. Such a remarkable change of function must rest upon some experimental error of the author.

colon group which may be divided into a saprophytic and a parasitic sub-group as follows:

A. Saprophytic sub-group.
  1a. Ferment all three sugars with same rapidity. . . Bacillus of grouse disease and some colon bacilli.
  1b. Ferment glucose and lactose rapidly, saccharose slowly. . . . *B. coli* α (1a and 1b).
  2. Ferment glucose and lactose rapidly, saccharose not at all. . . . *B. coli* β.

B. Parasitic sub-group.
  1. Ferment glucose rapidly, saccharose and lactose not at all. . . . all pathogenic forms.

I am inclined to associate this loss of functional activity in the pathogenic group B with an adaptation to a more parasitic existence and the development of certain other powers—the formation of toxic substances perhaps—which enables them to live in competition with living tissues while they have largely forfeited their power to compete with the more saprophytic forms from which they may have originally sprung.

It might be claimed that the phylogenetic loss of gas production is simply a change in the kind of fermentation, from the butyric to the lactic for example. That this is not true can be readily demonstrated, for in saccharose and lactose bouillon, when muscle glucose is absent and no gas appears in consequence, the reaction of the bouillon does not become acid. Among those bacteria which act upon sugars without the development of gas, a strongly acid reaction appears within twenty-four hours. The failure of the group B to act upon lactose is furthermore shown by their inability to produce coagulation of milk. We have, therefore, no ground for assuming a change in the type of fermentation. It is an absolute loss of function and not a modification.

In bringing together the more detailed observations on gas production a certain number of interesting facts claim our attention. We note that in the *B. coli* type of gas production in glucose only a certain quantity of gas collects—45 to 60 per cent. of the capacity of the closed branch—while in the *B.*

cloacæ type fully 100 per cent. is formed. Again the fraction $\frac{H^*}{CO_2}$ for *B. coli* is approximately $\frac{2}{1}$ while that for *B. cloacæ* is $\frac{1}{2}$ or $\frac{1}{3}$. The reaction of the fluid in cultures of the latter is feebly acid while for the *B. coli* group it is always strongly acid. Grimbert[22] in his studies of an anaërobic organism ascribes the greater production of CO_2 to a greater formation of alcohol and the more abundant production of H to a greater formation of acid in accordance with the following formulæ :

$$C_6H_{12}O_6 = C_4H_8O_2 + 2CO_2 + 4H \therefore \frac{H}{CO_2} = \frac{50}{50}$$

$$C_6H_{12}O_6 = C_4H_{10}O + 2CO_2 + H_2O \therefore \frac{H}{CO_2} = \frac{0}{100}$$

This would agree well with the feebly acid reaction of cultures of *B. cloacæ* and the strongly acid condition of those of *B. coli*.

Another phenomenon constantly observed is the great predominance of H over CO_2 in either type when only a little gas has been formed as in peptone bouillon containing traces of muscle sugar. The same phenomenon is noticeable when the gas at different stages of the process is examined. This may be illustrated by the three following stages in the gas production by *B. cloacæ*.

After 22 hours 37.5 per cent. gas has accumulated ; CO_2, 46.6 per cent. ; H, 53.4 per cent.

After 22 hours† 73 per cent. gas has accumulated ; CO_2, 61 per cent. ; H, 39 per cent.

After 96 hours 95 per cent. gas has accumulated ; CO_2, 70 per cent. ; H, 30 per cent.

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* We should not ascribe more than a comparative value to this fraction for the reason that Co_2 is much more soluble in water than H. Thus at 20° C. one volume of water takes up 0.9014 volumes of CO_2 and only .0193 volumes of H. If we bear in mind that at the beginning of fermentation a comparatively large quantity of CO_2 may become absorbed in the bouillon the relation of CO_2 to H in the fermentation tube will be understood to be entirely different from the ratio obtained by exact analytical methods.

† A second tube inoculated with the first but having produced gas more promptly.

I have been inclined to ascribe this to the absorption of CO_2 by the bouillon but Grimbert[22] as well as Frankland[17] finds by exact quantitative determinations of the gases the same increase of CO_2 as the fermentation progresses. The former explains it by assuming a greater production of alcohol in the later course of the process in accordance with the formulæ given above. According to this explanation the type of fermentation of *B. cloacæ* may differ from that of *B. coli* simply by an increased production of some alcohol at the expense of an acid. If we go a step farther and bring within the range of comparison another type of gas production, that of ordinary yeast by which only CO_2 and ethyl alcohol are produced (if we neglect traces of succinic acid) we have eliminated both the hydrogen and the acid element which seem to go together.

A farther point of interest is the constant presence in all cultures examined of an inflammable, explosive gas which I have assumed to be hydrogen. Most observers, including Arloing[4], Escherich[9], Frankland[17], Peré[7], Scruel[13], Grimbert[22], and others report only CO_2 and H. Baginsky[12] on the other hand claims the presence of CH_4 as well. It would be interesting to determine whether bacterial fermentation ever goes on without the evolution of both CO_2 and H at the same time.

In examining the action of bacteria on the three sugars used, we note that the gas production in glucose bouillon is always rapid though it may be slow or absent in lactose and saccharose bouillon. Glucose is thus the sugar most easily acted upon. A curious preference is shown by certain species for certain sugars. Thus *B. coli* produces gas rapidly in lactose and slowly or not at all in saccharose bouillon. Friedländer's bacillus on the other hand, acts vigorously upon saccharose and very slightly upon lactose. The latter is not touched by *Proteus vulgaris* at all. By pushing such comparative inquiries still farther and including other carbo-hydrates, as has been done by most of the authorities cited above from a slightly different point of view, still finer lines of distinction might be drawn. Owing to lack of time I have not

gone beyond the three sugars noted excepting to test some species in potato starch suspensions several years ago.*[16]

The source of the two gases CO_2 and H may be explained by the old formula of the text-books which splits one molecule of grape sugar into two of lactic acid and these into one of butyric acid and two each of CO_2 and H. This formula demands equal volumes of these gases. Scruel holds that the molecule of glucose breaks up into one of formic, of acetic, and of lactic acid with fixation of one atom of O. The gases he derives from the direct decomposition of the newly formed molecule of formic acid :

$$CH_2O_2 = CO_2 + H_2.$$

As has been recently emphasized by Grimbert and stated above, the process of fermentation varies from beginning to end so that no single equation can express more than what is going on at any one time. The same author ascribes this continual change to a modification of the vitality of the ferment organism brought about by the accumulation of harmful products in the fluid.

The rapid evolution of gas in the presence of one kind of sugar and its slow accumulation in the presence of another brings up the question whether or not an inverting ferment comes into play in the slow fermentation. This question is not approachable by the simple methods I have employed. It is certainly a curious fact that one bacterium may produce gas with almost equal rapidity in three sugars, another in two and that these two may be, with one species, glucose and saccharose, with another, glucose and lactose. Thus the bacillus of grouse disease produces gas in glucose, lactose and saccharose with equal rapidity.

Bacillus coli produces gas in glucose and lactose with equal rapidity. Action on saccharose variable.

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*The action of bacteria on potato starch may be demonstrated by cutting potatoes so that they fit rather snugly into test tubes. The film of water between them and the glass imprisons any gas bubbles that may be set free. In this way I noted the evolution of gas in several species, among them Friedländer's bacillus.

The bacillus of Friedländer produces gas in glucose and saccharose with equal rapidity. Very slight action on lactose.

Proteus vulgaris produces gas in glucose and saccharose with equal rapidity. No action on lactose.

Bacillus cloacæ produces gas in glucose and saccharose with equal rapidity. Action on lactose slow.

The probability of the direct breaking up of the molocule of saccharose and lactose[5] without inversion, has been affirmed by nearly all recent authorities and seems plausible when gas accumulates very rapidly as in cultures of *B. cloacæ*. It is evident that the observatious made with the fermentation tube open some very interesting problems, the solution of which must be left to others.

In connection with the selective action on sugars manifested by different species seemingly related to each other the thought has occurred to me that a clue to the habitat of bacteria might be obtained by an investigation of their predilections. Inasmuch as there are certain products such as lactose peculiar to animals, and certain others, such as saccharose peculiar to plants an adaptation to one or the other carbohydrate would indicate a saprophytic existence on animal or vegetable products. This hypothesis however needs a larger array of facts than I am able to put together, to prove or disprove its correctness.

The production of CO_2 and H together with other gases during the decomposition of proteid substances has been affirmed by Kerry[23] and Bovet[24]. The former used carefully prepared serum-albumin, the other serum-albumin and yolk of eggs. In the accurate determination of the source of gas production in putrefactive processes, it is evident that carbohydrates must be carefully eliminated since the fermentation of these substances with evolution of CO_2 and H seems to be such a wide spread function among bacteria.

There is one other phase of the subject of fermentation which has an important bearing upon bacteriology. I refer to the formation of acids* which seems to be clearly traceable

* Thus in milk cultures of *B. coli*, Baginsky[12] found formic, acetic, and lactic acids. The same were found by Scruel. Peré[7] detected acetic and lactic acid. Frankland, Stanley and Frew[17] determined, in cul-

to the presence of carbo-hydrates. Some years ago, Petruschky[25] examined the acid and alkali-producing functions of bacteria by using as a culture medium specially prepared whey from milk. I called attention to the fact that such classification had only a limited value since it depended entirely on the composition of the culture fluid[9]. The whey, having as an important ingredient, lactose, would prove only such bacteria acid-producing which were able to cause fermentation of the milk sugar while those which could not do this would show themselves as alkali producers. Bearing on this subject are the statements made by bacteriologists in the early days of this branch of biology that cultures of many bacteria are at first slightly acid before becoming alkaline. I suggested that this was probably due to traces of sugar in the culture fluid and I was able to prove this by causing an oscillation from an acid to an alkaline reaction and back again by adding at intervals small quantities of glucose to the bouillon. The alkali formed during the multiplication of bacteria was neutralized by the acid derived from the fermentation of the glucose. If this was small in quantity the acid or acids were formed in correspondingly small quantities and the alkaline reaction soon reappeared. I was able to show furthermore that the addition of small quantities of fermentescible sugar greatly favored the multiplication of bacteria by keeping down the alkaline reaction. After I began testing peptone bouillon for muscle glucose with gas-producing bacteria, I found that in bouillon free from sugar the multiplication of various acid producing bacteria such as streptococci, staphylococci, *B. typhosus*, *B. diphtheriæ*, *B. coli*, and *B. choleræ suis* is not attended with any acid reaction, either temporary or permanent.

So far as my observations have gone they show that all bacteria are alkali producers in bouillon free from carbo-hydrates, and that when one or the other of this group is present a very large number of the most easily cultivated bacteria are acid producers. This two-fold activity probably serves a useful purpose in keeping the medium in which they live more or

tures of the bacillus of Friedländer ethyl alcohol, acetic acid and a little formic and succinic acid. Grimbert[22] detected among the products of *B. orthobutylicus* normal butyric alcohol, butyric and acetic acid.

less neutral and therefore favorable to their continued multiplication. A good illustration of this fact is afforded by the growth of *B. coli* in saccharose bouillon. The gas production goes on (with most varieties) very slowly. The fluid in the bulb in contact with the air becomes alkaline and very turbid with growth. The fluid in the closed branch becomes acid under the influence of the slow fermentation and remains so. As it is gradually pushed out into the bulb by the slow accumulation of gas it tends to reduce, by degrees, the alkalinity of the fluid therein contained and thus favors step by step, the growth which finally becomes very dense.

The employment of sugar as a constituent of culture media is therefore, a matter of considerable importance. For certain species, like *B. coli* for instance, the addition of 1 per cent. glucose or lactose would be a decided detriment to the culture and soon lead to its destruction. Cane sugar on the other hand, added in the same proportion, would favor the growth owing to its much slower decomposition. Again the addition of very small quantities of glucose from time to time is favorable as stated above. In fact, bouillon, entirely free from muscle glucose, is less desirable than that containing traces, and in general it would be well to add glucose to bouillon. The limit may safely be put at 0.1 per cent. These remarks apply equally well to the large group of bacteria which produce acids in sugar solutions without the evolution of gas and in searching for the most favorable media for any species its behavior toward the more common carbo-hydrates should be carefully looked into.

APPLICATION OF THE FERMENTATION TUBE TO PROBLEMS IN PRACTICAL SANITATION. THE GAS TEST IN THE DIFFERENTIATION OF B. TYPHOSUS FROM THE B. COLI GROUP OF BACTERIA.

The use of the fermentation tube as an important differential test in bacteriology led me in 1889 to compare the frequently confounded species, *B. typhosus* and *B. coli communis*. A sharp distinction was at once detected between them which manifested itself by a total lack of gas production on the part

of the typhoid bacillus. In a brief article on the uses of the fermentation tube published in 1890[2], I incidentally called attention to this difference as a valuable means of diagnosis. The fact, however, remained unnoticed and in 1891 Chantemesse and Widal[26] brought forth the same test as new, using lactose in place of glucose in the bouillon. Their method consisted in observing gas bubbles rising and forming a light froth on the surface of the culture fluid in ordinary flasks. This publication induced me to defend my priority in a second article in which I quoted the original announcement of the test[27]. But even this has been largely overlooked by subsequent writers.

The publication of Chantemesse and Widal first called general attention to the gas test as the older differential characters were melting away and something more definite was urgently needed in this very practical field. They were opposed at once by Dubief[28] who regarded the differences between these species as merely quantitative. Recently a number of writers (Tavel[29], G. W. Fuller[30], W. Dunbar[5], Germano and Maurea[31], Ferrati[32], and Pane[33],) have contributed long articles on this subject and all of them confirm the gas test and give it the most important place among the means of diagnosis between *B. typhosus* and the colon group. Dunbar in ignorance of my second article * naïvely recommends the bent tube, closed at one end, as the simplest means of determining gas production. The same thing had been suggested by G. W. Fuller in a prior publication as a substitute for the more expensive fermentation tube. Dunbar further recommends simple bouillon (*Fleischwasser*), a recommendation likely to lead astray as I have pointed out above. Since gas production in bouillon depends solely on the muscle glucose the test would fail when this is absent. The use of lactose, as suggested by Chantemesse and Widal is not so trustworthy as that of glucose, for we have a large group of pathogenic bacilli, the hog cholera group, easily confounded with *B. typhosus* because neither act on lactose and hence do not coagulate milk. The use of glucose bouillon would clear up the difficulty at once.

* This must have appeared before the conclusion of his work for he refers to a publication subsequent to mine.

The fundamental differences between *B. typhosus* and the colon group of bacteria need further elucidation by a thorough study of all the products of fermentation, as has been done by Dubief[28] and Peré,[7] but without concordant results as yet. For the typhoid bacillus likewise has a definite action on glucose, as has been shown by Brieger and recently by Peré. The latter has shown that when glucose is added to milk, it subsequently coagulates when inoculated by this organism. The action on glucose is moreover readily revealed by the markedly acid reaction of cultures in glucose bouillon. All that the gas test tells us definitely is that the colon bacteria act on glucose with evolution of a certain volume of gas, and that the typhoid bacillus acts upon glucose without the evolution of gas.

There is one question called up by the fermentation test which will require some attention. The evolution of gas with the simultaneous appearance of acids in the culture fluid might lead us to assume that at least some gas may have been set free from the Na_2CO_3 used to neutralize the bouillon. Yet by adding increasing quantities of sterile Na_2CO_3 solution to a series of fermentation tubes, I was unable to evolve any gas with the typhoid bacillus. It is not unlikely, however, that bacteria capable of setting free much acid may lead to the accumulation of a trifle of gas, not the product of fermentation, in strongly alkaline bouillon. In all cultures in which only small quantities of gas appear this possibility should be borne in mind.

THE QUANTITATIVE DETERMINATION OF FECAL BACTERIA IN WATER.*

The bacteriological examination of water in the interest of practical hygiene, has thus far suffered from the difficulty that the kinds of bacteria present are recognizable only when a disproportionate amount of labor is spent in isolating them. Occasionally bacteriological water analysis has taken a certain

* See the forthcoming Annual Report of the State Board of Health of New York for 1892, for a more detailed statement of this method.

definite direction, as in the search for typhoid bacilli and Asiatic cholera spirilla. For general purposes, however, the bacteriologist had to fall back upon the numerical estimation with no regard to any qualitative determination. The numerical estimate, taken by itself, is not satisfactory. It is true that in large surface waters, such as rivers, the number of bacteria is a very good index of the organic matter present, yet here one remains in doubt whether the bacteria are in the main from sewage or from decaying vegetable matter. Hence in the few instances in which I have had occasion to determine the hygienic character of a given water, I have endeavored to get some idea of the fecal bacteria present, in other words, the large group of colon bacteria which are such regular inhabitants of the intestines of man and of the domesticated animals and which are as good an index of sewage pollution as we can desire.

There are methods which enable us to isolate fecal bacteria from water, but they either do not give us any information concerning the number of such bacteria, or else this knoledge is obtainable only after much labor. Passing by these methods as not bearing on our subject, I will briefly refer to one which is an outgrowth of the observations on gas production in the fermentation tube.

If a series of such tubes containing glucose bouillon be inoculated, each with an equal but very small quantity of water and placed at once in the thermostat at 37° C., it will be noticed after one or more days, if the water is much polluted, that some contain gas. If, for example, one ccm. of water is distributed equally among ten tubes and of these, four subsequently contain gas, we may conclude that in one ccm. of this water there were four gas-producing bacteria. All gas-producing bacteria are not intestinal species, however. Hence we must try to eliminate those that are not fecal by the amount of gas present. Bringing together all the information obtained by cultivating a variety of bacteria in the fermentation tube, I have come to the conclusion that all tubes containing less than forty and more than seventy per cent. of gas are to be eliminated. The lowest limit drawn excludes *Proteus vulgaris*,

probably a putrefactive organism, pure and simple. The upper limit excludes *B. cloacæ*, which, in spite of its name, I cannot range among fecal bacteria. Between the limits of forty and seventy per cent. of gas are included all varieties of *B. coli*, the hog cholera group, *B. lactis aërogenes* and Friedländer's bacillus.

There are several objections which may be urged against this as against any approximative method. In the first place it does not include a large number of pathogenic species, among them *B. typhosus* and *Sp. choleræ Asiaticæ*. But, it may be answered, the object of the method is not to reveal all possible disease germs but to use the colon group as an index of pollution because, as I maintain, they must come directly from the digestive tract. The presence of *B. coli* even in small numbers is amply sufficient to make any water suspected.

In the second place it may be claimed that the evolution of gas may be either checked or augmented in the presence of a number of species in the same tube. This objection involves the rather broad subject of antagonism among bacteria, which cannot be discussed here. There are, however, a few facts which show the objection to be in the main pointless. In the thermostat only very few bacteria from water develop owing to the high temperature, so that rarely more than one species survive and multiply in the fermentation tube if the quantity of water added be not too great. Again, the presence of two gas-producing bacteria in the same tube is not likely to occur owing to their relative scarcity. To test their mutual behavior, however, I inoculated a number of tubes simultaneously with two different gas-producing species. In general *B. coli* produced the quantity of gas peculiar to it, unless *B. cloacæ* was inoculated with it. In one out of three trials *B. cloacæ* triumphed and drove out all fluid from the closed branch, in the other two *B. coli* conquered. There may, therefore, be an occasional masking of the presence of *B. coli* by *B. cloacæ*. This error will not occur if small quantities of water be used or if the experiment be repeated in the event that more than half the tubes inoculated show gas production.

The concurrence of the many aërobic bacteria with the colon group in the fermentation tube even if they should be able to multiply at the temperature of the thermostat is made negative by the fact that the former are unable to multiply at all in the closed branch.

WASHINGTON, D. C.,
July 28, 1893.

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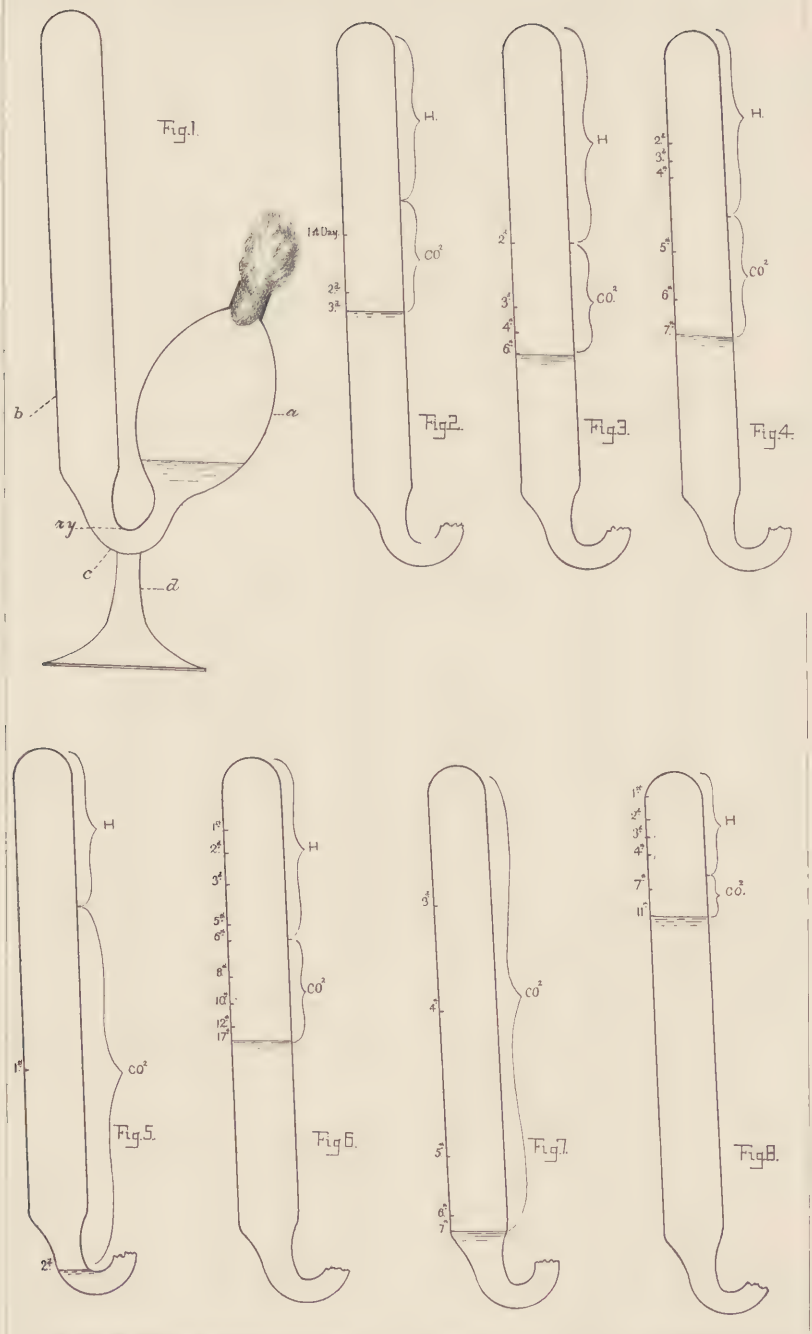
Fig.1.
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Fig4.
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Fig6.
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Fig7.
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Fig8.

DESCRIPTION OF PLATE.

(All figures reduced one-half.)

Fig. 1. The fermentation tube as used in the foregoing investigations. *a*, The bulb freely exposed to the air filtering through the cotton wool plug; *b*, the closed branch; *c*, the connecting tube; *d*, the foot. The tube used in the foregoing investigations requires about 25 cc. of bouillon, 20 of which belong to the closed branch. The line *xy* divides the aërobic from the anaërobic portion of the tube. This line is very sharply drawn by aërobic bacteria. The turbidity on the one side bounds absolute clearness on the other. In facultative anaërobic cultures there exists at this line a sudden marked change from turbidity to mere cloudiness.

Fig. 2–8. Graphic representation of gas production by different bacteria in different sugar solutions. The short lines on the left margin of the tube show the rapidity with which gas accumulates and serve as a means of comparing different types. The volume of CO_2 and H found at the close of the period of gas production is indicated by brackets on the right margin of the tube.

Fig. 2. *B. coli communis* in glucose bouillon.

Fig. 3. The same bacillus in lactose bouillon.

Fig. 4. The same bacillus in saccharose bouillon.

Fig. 5. *B. cloacæ* in glucose or saccharose bouillon.

Fig. 6. *B. cloacæ* in lactose bouillon.

Fig. 7. *Saccharomyces cerevisiæ* (isolated from compressed yeast) in glucose or saccharose bouillon.

Fig. 8. *B. coli* in peptone bouillon. The gas formed indicates the presence of considerable muscle glucose.

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